HYBRIDIZATION AS A TECHNIQUE OF BIOLOGICAL EVOLUTION

W. Frank Blair

The University of Texas at Austin.

INTRODUCTION

The hybridization of two species represents a method of more importance in obtaining the understanding of the relationships of these species. The crosses *in vitro* avoid the operation of the mechanisms that are aislated, as the characteristics of the mating call and the size of the body.

The results of these crosses provide an indication of the degree of affinity of the species. It is possible, naturally, that a singular gene can provide an obstacle for the crossing of two species, but the results of numerous crosses indicate that many genes involve themselves in the obstacles of crossing of the two species. Consequently, the results of the crosses permit the reconstruction of the evolutionary history of the groups. No method is of more importance with respect to this type of information than the hybridization method.

THE HISTORY OF THE TECHNIQUE OF HYBRIDIZATION

Use of the technique of hybridization as a tool in evolutionary studies of anuran amphibians was initiated about 1940. Investigation of the toads *(Bufo)* were begun by A. P. Blair (1941) and of frogs *(Rana)* by Moore (1941 and numerous subsequent publications). Moore (1955) also published a general review of the hybridization studies of anuran amphibians. My own published works with *Bufo* involve for the most part mostly

North American species (1959, 1961, 1963a), the *americanus* group (1963b), the *boreas* group (1964) and the *valliceps* group (1966). However, extensive information about hybridization of toads of all continents will be published in a forthcoming multi-authored book. Hybridization has been used to study the evolutionary relationships of Hylidae (Moore, 1955; W. F. Blair, 1958; Johnson, 1959; Littlejohn, 1961; Mecham, 1957, 1960a, 1960b; Pyburn and Kennedy, 1960, Pyburn 1960, and others). Hybridization of spadefoots *(Scaphiopus)* has been done by Wasserman (1957, 1958). Hybridization of Australian Leptodactylidae of several genera has been accomplished by Moore (1955).

METHODOLOGY

Hybridization of different species of anurans is relatively simple since the discovery that it is possible to stimulate ovulation by intraperitoneal or intramuscular injection of whole anuran pituitary glands or of purified mammalian chorionic gonadotrophins (Rugh .....). The latter function well in some species groups of *Bufo* (e.g., the *americanus* and *valliceps* groups) but in many other toads and in many other anurans they are ineffective. Consequently, the use of whole anuran pituitaries is recommended for induction of ovulation in most species. In my experience, two injections are necessary when females that have been a long time in the laboratory are used. If the first injection does not induce ovulation, we wait forty-eight hours before making a second injection. This second injection usually induces ovulation, but if it is unsuccessful, a third or even fourth injection at forty-eight hour intervals may produce results.

Once a female starts to ovulate, the males to be crossed with here are sacrificed by pithing. The testes are crushed in a small amount of unchlorinated water, and the eggs are squeezed into this sperm suspension. The eggs are best agitated for the first few seconds after they enter the solution to provide the highest probability of being fertilized. A single large female may be crossed with a large number of males by using only a few hundred eggs for each cross. One male can be used to fertilize the eggs of several females that are ovulating at the same time. The testes may be split so that one is used to fertilize the eggs of one female and the other to fertilize those of another female. Large testes may be separated into several parts, each of which can be used with a different female. Even with small testes, the sperm suspension may be decanted off once or twice to fertilize the eggs of a second and third female. A control fertilization should be made by putting some of the eggs into a sperm suspension of the same species. In the sometimes absence of a male of the same species a satisfactory control can be accomplished by using a male that is known to produce viable hybrids with the species of female in question.

As soon as cleavage lines can be seen, a sample of usually 100 to 200 eggs is separated out of each set, and the total number of eggs in the sample and the number of cleaving eggs are recorded. This group of eggs is referred to as the "count-sample". The count samples are examined again at age about twenty-four hours, and abnormalities of development, such as exogastrulation are recorded. After the larvae have hatched, the number that hatch in the count sample is recorded and notes are made with respect to obvious abnormalities. These counts give us the information as to what percentage of the fertilized eggs resulted in a hybrid embryo that was sufficiently normal to hatch into a motile larva. It should stressed that in the anura the percentage of the eggs that are fertilized carries no information of evolutionary significance, with one special exception to be discussed later. However, it does provide information with respect to the freshness or staleness of the eggs. Once the process of ovulation is initiated, the eggs will deteriorate in a matter of hours or days, depending on the species, if the eggs are not laid. Thus, a set of eggs from a female taken in amplexus in the field usually shows some percentage of stale eggs unless the eggs are stripped within a few hours.

After the larvae are hatched, they are put into subsamples, with 20 individuals per enamel pan of the dimensions of approximately 320 mm in length, 240 mm in width, and 60 mm in depth. The count sample is also put into these pans, with no more than 20 larvae per pan. If the size of the stock permits, 10 pans of 20 each are set up in addition to the count sample. A card is attached to each pan, and on this a record is kept of the number metamorphosing each day, of the number found dead each day and of obvious abnormalities. By compilation of these records it is possible to determine the number of fertilized eggs that resulted in metamorphosed toads in each set.

Larval *Bufo, Hyla* and Microhylidae have been raised in my laboratory solely on a diet of boiled lettuce. The amount of lettuce put into the pans daily is gauged so that most will be eaten. Any uneaten lettuce and fecal materials should be removed daily as fresh lettuce is being added. In this way, no change of water is required and it is necessary only to replace that lost by evaporation.

As soon as a young metamorphosing individual frees one or both forelegs, it is placed in a sloping pan with water at the end with a cloth cover. After the tail is resorbed, the frog is placed on dirt in a covered aquarium with others of its kind. These are given a diet of *drosophila* in excess quantities. The young and adults of terrestrial isopods *(Armadillidium)* have been found excellent for some species of *Bufo* as the young toads become too large for efficient feeding *Drosophila* but are still too small for feeding on beetle larvae *(Tenebrio)* which provide our standard food for adult toads. However, some species of *Bufo* refuse to eat the isopods.

The hybrid toads are raised to maturity and tested for fertility whenever possible by back-crossing to the parental species.

The technique of "haploid hybridization" of Moore (1960a, 1960b, 1967a, 1967b) should be mentioned. This involves the transfer of nucleus to foreign enucleated egg. Moore (1964) places great significance on this method as a measure of evolutionary divergence. I am unable to agree, as he is measuring only the ability of the nucleus of one species "to find a suitable biochemical environment in the cytoplasm of another". Moore's work has shown generally that this method enhances abnormalities that he obtains in diploid hybrids of *Rana.*

RESULTS

The results of the crosses range all the way from essentially absolute fertility of both the male and female hibrid F_1, to the failure of the zygote to develop any further than gastrulation. The results form a virtual sequence, but some levels predominate in the following way (Blair, ms):

1. Failure at the moment of gastrulation, with or without exogastrulation.
2. Failure as a monster in the early stages of tail-appearance.
3. Failure as abnormal larva (Microcefalic, edematous or stout) that are incubated but never eat.
4. Failure as an apparently normal larva, that eat and grow, but after several days apparently another essential requirement is lacking for additional development and it dies. This phenomenon is generally restricted to the large group of the African *regularis.*
5. Incapability for metamorphosis.
6. Metamorphosis of abnormal individuals that remain small, poorly colored, or lacking one or both eyes or one or more members of the body.
7. Metamorphosis of vigorous hybrids, at times heterotonic, but steril. The gonads at times are underdeveloped, abnormal, or apparently normal. There are at times testes well developed, so much as a bidders organ that contains pigmented eggs.

8. Hybrid adult males that produce great quantities of abnormal sperm, which fertilizes eggs producing a generation of unchangeable cross-mating.
9. Low percentage of F_1 fertil of one or the other sex, that can produce changeable cross-mated individuals.
10. High percentage of F_1 fertil of both sexes, that can produce many individuals changeably cross-mated.

INTERPRETATION OF RESULTS

The technique of hybridization provides information about the degree of affinity of the species of a genus that is probably unequalled in accuracy by the information that is available from any other currently feasible technique. The development of the hybrid to a particular stage provides information that the two parental species are sufficiently similar genetically to produce an offspring that is viable to that particular stage of development (e.g. to larva, or to metamorphosed anuran).

Hybridization does not give a measure of the total similarity or dissimilarity of the genotype of the two hibridized species. It merely measures the similarity in those fundamental processes and biochemical pathways necessary to produce a viable individual. Two species can, in some instances produce a viable F_1 hybrid even though they obviously differ in numerous genes that affect external appearance.

Many hibrid combinations fail to develop beyond the stage of gastrulation. This is the stage at which the male chromatin becomes involved in development, so failure at this stage is usually indicative of great dissimilarity of the two genotypes.

Extensive and extreme exogastrulation (failure of yolk to be incorporated in the developing embryo) is common in crosses of distantly related species. These exogastrulated embryos in some instances may precede on to become very abnormal exogastrulated tail-bud larvae. In other combinations, the embryos fail at gastrulation without exogastrulation.

In general, the stage to which development progresses (that is, the genetic compatibility) is, crudely at least, a function of the degree of affinity as indicated by other kinds of evidence, e.g. by morphology. However, there are interesting exceptions or the use of this technique would not be justified.

We feel that the information from hybridization experiments should be used only as one of many kinds of data. Any absolute reliance on the results of hybridization without reference to other kinds of information could lead to erroneous conclusions.

For example, relatively recently evolved species, members of the same species group, with affinity obviously attested to by morphological similarity, are usually infertile in the genus *Bufo*. However, in the *Bufo regularis* in Africa two broadly sympatric species that have been hopelessly confused by taxonomist until very recently are unable to form metamorphosable hybrids, although the hybrids reach the larval stage in both reciprocals of the cross. In the same group, there is at least one other instance of two apparently good biological species that are presently inseparable on morphological grounds, but they show a high level of genetic incompatibility. In a converse kind of situation, three of 11 hybrid males from the cross ♀ *B. woodhousei* x ♂ *B. arenarum* showed low levels of fertility, although the reciprocal hybrids were inviable. All other evidence points to the *americanus* group to which *B. woodhousei* belongs and *B. arenarum* as being long-separated and relatively distantly related parts of the new world radiation of *Bufo*. To attribute great weight to the fertility of these F_1 males as against all of the other kinds contrary evidence would lead to erroneous conclusions as to affinity. Actually, the examples we have just given are rare, and with few exceptions the data from hybridization experiments and from other methodologies tend to support each other strongly and hence give confidence in the conclusions about evolutionary relationships that can be drawn from the combined data.

Experience has provided a number of cautions that should be exercised in the interpretation of the results from hybridization. Some of the most important ones are:

(1) Reciprocal interspecific hybrids may show very different levels of compatibility, which is, of course, a reflection of differences in egg cytoplasm. Therefore, it is important to attempt to produce both reciprocals, and each reciprocal can give its own kind of information. For example, the females of the *Bufo americanus* group will form viable hybrids with males of many other kinds of *Bufo*, hence failure of an F_1 hybrid that has an *americanus* – group mother is significant of distant relationship. Males of the *americanus* – group conversely are highly incompatible with the females of most other *Bufo*, so a combination in which hybrid larvae fathered by an *americanus* – group male are viable is indicative of closeness of relationship.

(2) In *Bufo* at least, there has evolved at least twice a hypertrophy of the testes. Sperm suspensions from such testes inhibit fertilization and/or development of heterospecific eggs. In this instance, development usually

fails prior to gastrulation. Hybrid experiments involving these males obviously cannot give the same kind of genetic information provided by crosses that involve males that lack this specialization, because the action of the presently unidentified chemical agent in the enlarged testes determines the outcome of the cross. Nothing comparable has been reported in any other group of anurans.

(3) It is necessary to gain an understanding of the general pattern of interspecific compatibility in a genus before drawing evolutionary conclusions from the results of hybridization experiments. This can be illustrated by comparing the genera *Bufo* and *Rana*. As my work has demonstrated, species of *Bufo* which clearly could have had no genetic interchange for millions of years have retained genetic similarity that they can produce viable, but typically sterile, hybrids. In the genus *Rana* as demonstrated by Moore (?) and various others viable hybrids can be produced only among the very closely related members of the same species group. The results of hybridization experiments must be interpreted quite differently in the two genera, and, in fact, hybridization is of virtually no use in tracing distant relationships of *Rana* species. The genus *Hyla* is comparable to *Bufo* rather than to *Rana* in this *respect*, which makes possible the same kind of investigation of the tree frogs that has been under way with *Bufo*.

(4) It is highly desirable that the karyotype be determined, especially in crosses in which very few larvae hatch. Bogart (ms) has found that diploid eggs are more common in *Bufo* than previously thought, and triploid and even pentaploid hybrids have been found.

Because these have a diploid, or tetraploid contribution of maternal genetic material which could control development without influence from the haploid male contribution, the survival and development of these triploid or pentaploid hybrids is of dubious value in indicating degree of affinity. Bogart (op. cit.) has found one instance of gynogenetic diploidy (a double set of female chromatin). Survival of these hybrids could have led to erroneous conclusion if the karyotypes had not been examined. Bogart (op. cit.) has found no evidence of polyspermy, nor has he ever found a haploid larva. If haploid individuals begin development in *Bufo*, they apparently fail prior to hatching.

(5) The condition of the eggs should be taken into account. In some instances only a few eggs in a set remain fresh enough to be fertilized but then proceed to develop normally. In other instances, these few fertilized eggs will fail at some stage although fertilized fresh eggs of the same combination are known to develop through hatching and metamorphosis.

Crosses of this kind should be repeated if possible, and if this is not possible, they should be interpreted with caution.

APPLICATION OF THE DATA TO EVOLUTIONARY PROBLEMS

In genera such as *Bufo* and *Hyla,* in which viable hybrids can be formed by rather distantly related species, the data from hybridization provide a powerful tool for revealing evolutionary relationships. I will cite only two examples from the *Bufo* work.

Bufo viridis, with its close relative *B. raddei* of Asia range across Eurasia and into North Africa. *B. viridis* has been crossed with members of 6 African species groups, 16 North American groups and 5 South American groups (Blair, ms.). When one examines the results, it is apparent that narrow-skulled toads that are associated with the mountains of western North and South America show varying degrees of compatibility with *B. viridis,* while broad-skulled toads of South American lowlands and various groups of North American toads do not. Furthermore, the greatest compatibility is with certain toads of western North America. When other evidence is considered, it becomes apparent that the hybridization data have revealed an ancient dispersal route of narrow-skulled toads. Other hybridization data which will not be discussed here, lend a part of the support of the belief that the dispersal was from a point of origin in the new world, probably in South America.

In another instance, the hybridization data indicate that affinity rather than convergence accounts for the occurrence of broad-skulled toads in Africa *(regularis* group), southern Asia *(melanostictus* group) and in South America and southern North America. Here again the certainty of an ancient dispersal is indicated, this time involving the warmth-adapted broad-skulled toads.

Discovery of these relationships would have been even more unlikely without use of hybridization experiments. The line is absent today from the middle latitudes of North America and Eurasia, presumably having been eliminated there by cool Pliocene or Pleistocene climates. Furthermore, the African representatives have lost a pair of chromosomes (Bogart, 1968).

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